Tol-Pal proteins are critical cell envelope components of *Erwinia chrysanthemi* affecting cell morphology and virulence

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The *tol-pal* genes are necessary for maintaining the outer-membrane integrity of Gram-negative bacteria. These genes were first described in *Escherichia coli*, and more recently in several other species. They are involved in the pathogenesis of *E. coli*, *Haemophilus ducreyi*, *Vibrio cholerae* and *Salmonella enterica*. The role of the *tol-pal* genes in bacterial pathogenesis was investigated in the phytopathogenic enterobacterium *Erwinia chrysanthemi*, assuming that this organism might be a good model for such a study. The whole *Er. chrysanthemi* *tol-pal* region was characterized. Tol-Pal proteins, except TolA, showed high identity scores with their *E. coli* homologues. *Er. chrysanthemi* mutants were constructed by introducing a *uidA*–kan cassette in the *ybgC*, *tolQ*, *tolA*, *tolB*, and *tolF* genes. All the mutants were hypersensitive to bile salts. Mutations in *tolQ*, *tolA*, *tolB* and *pal* were deleterious for the bacteria, which required high concentrations of sugars or osmoprotectants for their viability. Consistent with this observation, they were greatly impaired in their cell morphology and division, which was evidenced by observations of cell filaments, spherical forms, membrane blebbing and mislocalized bacterial septa. Moreover, *tol-pal* mutants showed a reduced virulence in a potato tuber model and on chicory leaves. This could be explained by a combination of impaired phenotypes in the *tol-pal* mutants, such as reduced growth and motility and a decreased production of pectate lyases, the major virulence factor of *Er. chrysanthemi*.

INTRODUCTION

The Tol-Pal system of Gram-negative bacteria is required for outer-membrane stability (Lazzaroni et al., 1999). It comprises five envelope proteins, TolQ, TolR, TolA, TolB and Pal, which form two complexes. The TolQ, TolR and TolA inner-membrane proteins interact via their transmembrane domains (Derouiche et al., 1995; Lazzaroni et al., 1995). The β-propeller domain of the periplasmic protein TolB is responsible for its interaction with Pal (Ray et al., 2000). TolB also interacts with the outer-membrane peptidoglycan-associated proteins Lpp and OmpA (Cascales et al., 2002; Clavel et al., 1998). TolA undergoes a conformational change in response to changes in the proton-motive force (Germon et al., 2001), and interacts with Pal in an energy-dependent manner (Cascales et al., 2001). The C-terminal periplasmic domain of TolA also interacts with the N-terminal domain of TolB (Dubuisson et al., 2002; Walburger et al., 2002).

The transcriptional organization of the *E. coli* *tol-pal* genes has been characterized. The genes *ybgC* (orf1), *tolQ*, *tolR*, *tolA* and *tolB*, and *pal* and *ybgF* (orf2) form two operons (Muller & Webster, 1997); a large *ybgC–ybgF* transcript has also been postulated (Vianney et al., 1996). *YbgC* and *ybgF* encode proteins of unknown function located in the cytoplasm and the periplasm, respectively (Clavel et al., 1996; Sun & Webster, 1987). Inactivation of these two ORFs induces no obvious phenotype in *E. coli*. In contrast, mutations in the *tol-pal* genes cause the disruption of outer-membrane integrity, which is evidenced by several phenotypes, including release of periplasmic content, sensitivity to bile salts and other chemical compounds, formation of outer-membrane blebs at the cell surface, and overproduction of colanic acid (Bernadac et al., 1998; Clavel et al., 1996; Vianney et al., 1994). The *tol-pal* genes are also necessary for proper functioning of some uptake systems at the level of the cytoplasmic membrane (Llamas et al., 2003a). In *E. coli*, group A colicins and filamentous-phage DNA use the Tol proteins for their translocation across the cell envelope.
(Bouveret et al., 1998; Webster, 1991). The existence of tol-pal genes has now been established in several Gram-negative bacteria (Bowe et al., 1998; Dennis et al., 1996; Heilpern & Waldor, 2000; Llamas et al., 2000; Prouty et al., 2002; Youderian et al., 2003). There is also evidence that some of the tol-pal genes are involved in the pathogenesis of *E. coli* (Hellman et al., 2002), *Haemophilus ducreyi* (Fortney et al., 2000), *Salmonella enterica* (Bowe et al., 1998) and *Vibrio cholerae* (Heilpern & Waldor, 2000). However, the way that the tol-pal genes are involved in the pathogenesis of these bacteria is not well documented, except in *V. cholerae*, where tolQRA mutants show defects in the uptake of ctxΦ DNA, which encodes cholera toxin (Heilpern & Waldor, 2000), and in *E. coli*, where Pal initiates inflammation in sepsis, in synergy with lipopolysaccharide (Liang et al., 2005). The entero bacterium *Erwinia chrysanthemi* may be a good alternative to study the involvement of the Tol-Pal proteins in pathogenicity. It is responsible for soft rot in many plants, including vegetable and ornamental species. It colonizes parenchymatous tissues by degrading the plant cell wall by means of a battery of pectinolytic enzymes. The oligosaccharides originating from pectin degradation are used as a carbon source by the bacterium (Hugouvieux-Cotte-Pattat et al., 2001).

Analysis of the role of the tol-pal genes in the pathogenesis of such phytopathogenic species may be easier than in animal pathogens and could reveal additional properties of the Tol-pal proteins that may be difficult to observe in the *E. coli* K-12 laboratory strains. In addition, complementation studies and the use of hybrid proteins between the two bacteria may help in the understanding of some unresolved features of the Tol-Pal system. In a first attempt, the *Er. chrysanthemi* tol-pal genes were cloned and sequenced, and mutants in most genes were isolated and characterized.

### METHODS

**Bacterial strains, plasmids and growth conditions.** *Er. chrysanthemi* and *E. coli* derivatives are listed in Table 1. Plasmids used for subcloning experiments were pJEL250 (Valentin-Hansen et al., 1986), pBR328 (Soberon et al., 1980) and pKO3 (Link et al., 1997). Plasmid pN496 was a pBluescript derivative from the laboratory collection, containing a *uidA*–kan cassette flanked by multiple cloning sites.

Strains were grown in LB broth (with 5 g l⁻¹ NaCl) or M63 minimal medium at 37°C for *E. coli* or 30°C for *Er. chrysanthemi* (Miller, 1992). Tryptone swarm plates were used for motility assays and contained 3.5 g l⁻¹ Bacto agar (Difco), 10 g l⁻¹ Bacto tryptone (Difco) and 5 g l⁻¹ NaCl. The osmoprotectant glycine betaine was added to the growth medium at a final concentration of 1–10 mM. Antibiotics were added at a final concentration of 100 μg ml⁻¹ for ampicillin and 20 μg ml⁻¹ for kanamycin. Bacterial growth was monitored by OD₆₀₀.

**Transfer of RP4 derivative plasmids by mating.** pULB110, a kanamycin-sensitive derivative of RP4::mini-Mu, was used to generate R-prime derivatives containing bacterial DNA (van Gissegem et al., 1985). Mating between the recipient *E. coli* strain JC11305 and *Er. chrysanthemi* donor strains carrying plasmids was performed by spreading 0.2 ml of overnight cultures of the strains on M63 plates and incubating for 5 h at 30°C. Bacteria were resuspended in 1 ml of M63 medium and spread on selective media.

**Cloning and sequencing of *Er. chrysanthemi* tol-pal genes.** Most techniques were performed as described by Sambrook et al. (1989). For Southern blot analysis, DNA probes were prepared using the DIG High prime DNA labelling and detection starter kit (Roche). Recombinant plasmids were introduced into *E. coli* after a CaCl₂ treatment or into *Er. chrysanthemi* by electroporation at room temperature. Nucleotide sequencing was performed by Genome Express SA (Grenoble, France).

**Construction of *uidA*–kan insertions and marker exchange recombination.** The *ybgC*, tolQ, tolA, tolB, pal and *ybgF* genes were individually subcloned into pBR328 in order to generate plasmids with unique restriction sites in each gene. Two methods were used for marker exchange recombination. In the first one, the *uidA*–kan cassette of pN496 was introduced in the following unique sites: Msc

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**Table 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JC8056</td>
<td>supE44 hsdS metB1 gal lacY flnuA ΔlacU169</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>JC9776</td>
<td>8056ΔybgCtolQRA</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>JC11305</td>
<td>8056Δuid::Tn10 ΔtolBpal</td>
<td>Lab. collection</td>
</tr>
<tr>
<td><em>Er. chrysanthemi</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3937</td>
<td>Wild-type</td>
<td>Lab. collection</td>
</tr>
<tr>
<td>MC12001</td>
<td>A3937ΔybgC1::uidA–kan insertion at Msc after aa 8 of YbgC</td>
<td>This work</td>
</tr>
<tr>
<td>MC12002</td>
<td>A3937tolQ1::uidA–kan insertion at HindIII, removes the 27 C-terminal aa of TolQ</td>
<td>This work</td>
</tr>
<tr>
<td>MC12007</td>
<td>A3937ΔybgF1::uidA–kan insertion at EcoRV, after aa 69 of YbgF</td>
<td>This work</td>
</tr>
<tr>
<td>MC11634</td>
<td>A3937tolA1::uidA–kan insertion at HindIII, removes the 7 C-terminal aa of TolA</td>
<td>This work</td>
</tr>
<tr>
<td>JF11970</td>
<td>A3937tolQ2::uidA–kan insertion at NheI, after aa 13 of TolQ</td>
<td>This work</td>
</tr>
<tr>
<td>JF11971</td>
<td>A3937tolA2::uidA–kan insertion at NcoI, removes the 54 C-terminal aa of TolA</td>
<td>This work</td>
</tr>
<tr>
<td>JF11972</td>
<td>A3937tolB1::uidA–kan insertion at Hpal, removes the 59 C-terminal aa of TolB</td>
<td>This work</td>
</tr>
<tr>
<td>JF11973</td>
<td>A3937pal1::uidA–kan insertion at StuI, after aa 10 of the Pal signal peptide</td>
<td>This work</td>
</tr>
</tbody>
</table>
for ybgC, HindIII for tolQ and tolA, HpaI for tolB, SmaI for pal and EcoRV for ybgF (Fig. 1). In this cassette, the promoter region of uidA was absent, allowing the generation of a transcriptional fusion between the gene containing the insert and uidA. Plasmids containing the genes inactivated by the insertion were introduced into Er. chrysanthemi cells by electroporation. Integration of the insertions into the Er. chrysanthemi chromosome by marker exchange recombination was facilitated by prolonged culture in low-phosphate medium.
in the presence of kanamycin (Roeder & Collmer, 1985). Colonies recovered on kanamycin LB plates were analysed by replica plating on ampicillin LB plates to confirm the loss of the plasmid vector (referred to as method 1). In the second method, the *uid*Δ-*kan* cassette was inserted in the *Hind*III, *Smal*BI, *Hpa*I and *Stu*I sites of tolQ, *tolA*, *tolB* and *pal*, respectively. Fragments overlapping the insertion were cloned into the *Sma*I or *Not*I sites of pK03, a gene replacement vector that contains a temperature-sensitive origin of replication and markers for positive and negative selection for chromosomal integration and excision (Link et al., 1997). After electroporation, integration of the plasmid into the chromosome was selected by growth at 43°C in the presence of kanamycin, then integrates were resolved at 30°C and plasmid loss was selected in the presence of 10% sucrose. The colonies were then screened for sucrose resistance and the loss of chloramphenicol resistance by replica plating. For both protocols, the correct inactivation of the genes was controlled by PCR amplification of the chromosomal DNA, using the first primer in the *uid*Δ gene and the second primer in the *tol* gene. A control was also carried out to check for the absence of an intact gene.

**Motility assays.** Tryptone swarm plates were inoculated with a toothpick and bacteria were grown from 16 to 24 h at 30°C. The diameter of the growth area for each mutant was compared to that of the wild-type strain. Plates were inoculated with a maximum of four bacterial strains per plate, including the wild-type as a control.

**Phenotype analysis of mutants.** Sensitivity towards antimicrobial agents was tested on LB plates by replica plating the strains on rich media containing various amounts of the tested agent. To test strain virulence, 10^5 cells were inoculated in chicory leaves after scarification. After 24 h of incubation at 30°C, the length of the rotted region was measured. Potato tubers were inoculated as previously described (Lojkowska et al., 1995). A hole was made into the tuber parenchyma and 10^4 cells were used for inoculation. After 24–72 h incubation, tubers were sliced vertically through the infection point, and the weight of decayed tissue was taken as a measure of disease severity. All the macerated tissue was collected and used for bacterial counting. For each bacterium, 500 mg of macerated tissue was resuspended in 500 μl M63 medium, treated with 50 μl tolueone, and vortexed thoroughly. After centrifugation (12000 g for 2 min), the supernatant was used to determine β-galactosidase (which reflected the colony number, see Methods, Enzyme assays, below) and pectate lyase activities.

**Microscopy.** Bacterial cells were directly observed after growth in LB medium, LB medium supplemented with 10% sucrose or in chicory leaves. This allowed us to estimate the morphology of the cells. Bacteria were also recorded using a camera to estimate their motility.

For transmission electron microscopy, cells were grown on LB plates or LB plates supplemented with 10% sucrose, recovered with a loop on a Parafilm sheet, and then fixed with osmium tetroxide vapours. They were resuspended in 0.1 M sodium cacodylate, and then stained with 1% sodium silicotungstate or uranyl acetate.

**Enzyme assays.** Pectate lyase activity was measured in 0.1 M Tris, pH 8.5, 0.1 mM CaCl₂, 0.05% polygalacturonate, by following an increase in A230 from the cleavage of polygalacturonate. The assay for β-galactosidase activity has been described elsewhere (Miller, 1992). Because the cell morphology of the *tol*-*pal* mutants was heterogeneous, the OD₅₀₀ did not necessarily reflect the amount of bacteria. Thus, we calculated the relative natural β-galactosidase activity of *Er. chrysanthemi* based on colony number, which was determined by cell enumeration. Accordingly, the pectate lyase activity of each strain was divided by its β-galactosidase activity to standardize the enzyme activity (referred to as ‘relative units’ in the text). Cell enumeration was determined by plate count.

**Western blot analyses.** Cells (3 × 10^8) in the mid-exponential phase of growth were centrifuged, resuspended in loading buffer and boiled. Samples were separated by SDS-PAGE [12% polyacrylamide, (Laemmli, 1970)] and transferred for 2 h onto a nitrocellulose membrane by using a semi-dry blotter. Immunoblots were developed with the BM chemiluminescence blotting substrate (Roche). Polyclonal antibodies raised against *E. coli* TolA, TolB, Pal and YbgF proteins have been previously described (Clavel et al., 1998) and were used to detect the corresponding *Er. chrysanthemi* proteins.

**Nucleotide sequence accession number.** The nucleotide sequence reported here, corresponding to a 6967 bp fragment, has been deposited at EMBL under the accession number AJ297885 (EMBL/genebank version AJ297885.1 GI:16116629).

**RESULTS**

**Complementation of the ΔtolBpal mutation of *E. coli* with an RP4 derivative of *Er. chrysanthemi* A3937**

We used the selectable marker *nadA*, located 3 kb downstream from the *tol-pal* locus of *E. coli*, to clone the *tol-pal* region of *Er. chrysanthemi*, since the genetic organization of several genomic regions is quite similar in *E. coli* and *Er. chrysanthemi*. Plasmid pULB110 was used to generate R-prime derivatives containing an insert of bacterial DNA from *Er. chrysanthemi* that complemented the *nadA* mutation of strain JC11305 (*nadA*: *Tn10 ΔtolBpal*). Nad^+ transconjugants were isolated on M63 medium plates without nicotinic acid. Plasmid pR’16 was retained for further studies, since it complemented not only the *nadA* mutation but also the cholate-sensitivity phenotype associated with the ΔtolBpal mutation. Transfer of pR’16 in JC9776 demonstrated that this plasmid also complemented the cholate-sensitivity phenotype of the ΔybgCtolQRA mutation.

**Cloning and sequencing of *Er. chrysanthemi* tol-pal genes**

Plasmid pMC2242, containing an 8.5 kb EcoRI fragment of pR’16 cloned into pJEL250, complemented the cholate-sensitivity phenotype of the ΔtolBpal mutation of JC11305. DNA sequence analysis revealed that it contained the *ybgCtolQRA* fragment (Fig. 1). To clone the region upstream of *ybgC*, an EcoRI–*Smal*BI fragment from pMC2242 containing the *ybgCtolQRA* genes was used as a DNA probe for Southern analysis of pR’16 digested with HindIII. A 4.3 kb HindIII fragment was isolated and cloned into pBR322 to give pMC2244 (Fig. 1). Finally, a BamHI–*Hind*III fragment from pMC2244 was introduced into pMC2242 to give pMC2256. Further sequencing showed that it contained the entire *ybgC* sequence and its upstream region. The 6967 bp fragment cloned into pMC2256 is presented in Fig. 1(A). Upstream of *ybgC*, homologues of genes *cydB* (truncated), *ybgT* and *ybgE* of *E. coli* were identified. The region downstream from *ybgF* showed high similarity to *lysF*, indicating that the gene order in the *tol-pal* region of *Er. chrysanthemi* is the same as that found in *E. coli*. Unlike in *E. coli*, no potential transcription terminator could be
Gene inactivation

The first method used for gene inactivation allowed us to obtain mutants ybgC1, tolQ1, tolA1 and ybgF1, containing a uidA–kan cassette in the MscI, HindIII (for tolQ1 and tolA1), and EcoRV sites, respectively. The correct insertion and orientation of the cassette in the chromosome were confirmed by PCR. In the course of our experiments, we observed that the tolQ1 mutation was highly unstable; this mutation could not be retained. Moreover, attempts to inactivate the chromosomal alleles of the tolB and pal genes using this method were unsuccessful. We hypothesized that prolonged culturing in low-phosphate medium could be lethal for some of the tol-pal mutants. Hence, we used the second method (see Methods), which allowed growth in rich medium. Er. chrysanthemi was transformed with pKO3 derivatives containing tolQ, tolA, tolB and pal inactivated by the uidA–kan cassette in the NheI, SnaBI, Hpal and StuI sites, respectively (Fig. 1A). To our surprise, the colonies selected on LB plates supplemented with 10% sucrose grew very slowly on LB plates without sucrose. Therefore, we retained colonies which were able to grow on plates supplemented with sucrose and kanamycin but unable to grow on rich media supplemented with sucrose and chloramphenicol (loss of the pKO3 vector), or cholate (tol phenotype). Using this technique, we were able to obtain the mutants tolQ2, tolA2, tolB1 and pal1 (Table 1). The orientation of the uidA–kan cassette was investigated and shown to generate a transcriptional fusion between the inactivated gene and uidA in all but the tolA2 mutant. The poor growth of the tolB and pal mutants in the absence of sucrose provides a good explanation for our inability to isolate these mutants using the first method. The tolA1 mutant first isolated lacked only the seven C-terminal amino acid residues of TolA1, and comparison of the tolA1 and tolA2 mutants suggested that tolA1 retained a partial functionality. This could be due to the presence of higher amounts of TolB and Pal in the tolA1 mutant (Fig. 2). The instability of the tolQ1 mutant could also be explained by its poor ability to grow on LB plates.

Analysis of Er. chrysanthemi TolA, TolB, Pal and YbgF synthesis by Western blotting

The impact of the mutations on the production of the TolA, TolB, Pal and YbgF proteins was determined by immunodetection of the proteins. Cells were grown in 10% sucrose LB broth until mid-exponential phase. After separation by SDS-PAGE, Western blot analysis was carried out and polyclonal antibodies against the four proteins were used for immunodetection (Fig. 2). TolA, TolB, Pal and YbgF were missing in the tolA, tolB, pal and ybgF mutants, respectively. Many mutations led to the absence or a great reduction in the level of proteins encoded by downstream genes. For instance, both TolA and TolB levels were reduced in the ybgC, tolQ and tolA mutants. Pal was also reduced in tolA2 and tolB1 mutants. YbgF was reduced in most mutants, but particularly in the pal1 mutant. These results support the hypothesis of a large ybgC–ybgF transcriptional unit that could lead to a polar effect of the upstream mutations. However, since many Tol-Pal proteins are known to form complexes, the instability of some of them may also result from the lack of their interacting partner(s).

Er. chrysanthemi tol-pal mutants are impaired in cell motility and sensitive to ionic strength and osmolarity

The wild-type strain, as well as the ybgC1, tolA1 or ybgF1 mutants, was able to grow on LB and M63 plates. In contrast, the tolQ2, tolA2, tolB1 and pal1 mutants did not form colonies on LB or M63 plates, except in the presence of 10% sucrose. They also grew very slowly in LB liquid medium and when they were patched on LB plates. The role of sucrose was unexpected. Its addition affects the medium osmolarity, but it can also act as a membrane and protein stabilizer in adverse conditions (Crowe et al., 1988; Leslie et al., 1995; Molina-Hoppner et al., 2004). To discriminate between these two effects, the mutants were grown on LB plates supplemented with sucrose, various amounts of sugars, NaCl or the osmoprotectant glycine betaine.
Table 2. Motility of the Er. chrysanthemi tol-pal mutants

Swarm plates (with or without sucrose) were inoculated as described in Methods and grown at 30 °C for 24 h. Results are the mean of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swarm diameter (mm) with:</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No sucrose</td>
<td>Sucrose (10 %)</td>
</tr>
<tr>
<td>3937</td>
<td>43·0 ± 3·4</td>
<td>40·6 ± 3·4</td>
</tr>
<tr>
<td>ybgC1</td>
<td>21·6 ± 3·6</td>
<td>34·6 ± 3·9</td>
</tr>
<tr>
<td>tolQ2</td>
<td>8·0 ± 2·3</td>
<td>19·4 ± 4·8</td>
</tr>
<tr>
<td>tolA1</td>
<td>7·6 ± 1·1</td>
<td>13·4 ± 1·1</td>
</tr>
<tr>
<td>tolA2</td>
<td>10·0 ± 2·8</td>
<td>17·4 ± 2·3</td>
</tr>
<tr>
<td>tolB1</td>
<td>0</td>
<td>3·4 ± 0·5</td>
</tr>
<tr>
<td>pal1</td>
<td>0</td>
<td>5·0 ± 1·0</td>
</tr>
<tr>
<td>ybgF1</td>
<td>36·8 ± 2·9</td>
<td>38·2 ± 2·2</td>
</tr>
</tbody>
</table>

tolQ2, tolA2, tolB1 and pal1 mutants recovered a normal growth when sucrose was replaced by 10% glucose, galactose (two sugars metabolized by Er. chrysanthemi), maltose or lactose (two sugars not metabolized by Er. chrysanthemi), but not glycerol. Addition of the osmoprotectant glycine betaine (1–10 mM) also allowed growth of the mutants. In contrast, the tolQ2, tolA2, tolB1 and pal1 mutants did not grow when NaCl was added to LB plates at concentrations between 0·1 and 0·3 M. The presence of NaCl in the medium affects both osmolarity and ionic strength. Therefore, we concluded that the mutants were sensitive to high ionic strength and low osmolarity.

The motility of the mutants was tested on swarm plates, as described in Methods. After 20 h growth at 30 °C, the swarm diameter was 43·0 ± 3·4 mm for the wild-type strain. The motility of the ybgF1 mutant was not significantly affected, that of the ybgC1 tolQ2, tolA1 and tolA2 mutants was strongly reduced, while tolB1 and pal1 mutants did not move at all (Table 2). Addition of 10% sucrose to the swarm agar plates partially restored the motility of the mutants (Table 2).

As the growth rate of the mutant strains could affect their motility, a more direct observation of fresh bacteria was carried out by phase-contrast microscopy, and cell motility was recorded with a camera. The results were in agreement with those observed on swarm plates. The wild-type strain moved rapidly, while the tolQ and tolA derivatives moved much more slowly, and the tolB and pal1 mutants did not move at all (data not shown).

Morphological characterization of the Er. chrysanthemi tol-pal mutants

The cells were examined by electron microscopy after negative staining of the cells (Fig. 3). After the growth of patches on LB plates, the wild-type strain was rod-shaped with a mean size of 2·1 × 0·65 μm and presented long flagella (Fig. 3A). Under the same conditions, the tolQ2 mutant presented an altered morphology with short twisted filaments (Fig. 3B). This could be due to the presence of square poles at one end of the cells, where the poles tended to grow to one side rather than at the middle of the bacteria (Fig. 3C). Bacteria had a few, short flagella. The tolA2 mutant also formed short filaments and had a few, short flagella (Fig. 3D). The septum was not always at the middle of dividing bacteria, leading to cells of heterogeneous sizes (Fig. 3E). Vesicles could be observed, even at the septum (Fig. 3D). The tolB1 mutant also formed short filaments, but had no flagella (Fig. 3F). The size of the cells and the position of the septa were highly irregular. The pal1 mutant lacked flagella (Fig. 3G). Vesicles appeared at the cell surface as well as very large envelope excrescences.

When cells were recovered from LB plates supplemented with 10% sucrose, they were all surrounded by a capsule (data not shown). The altered morphologies identified after growth on LB plates were conserved, except that (i) the tolQ2 mutant formed short curved filaments with round poles, (ii) the tolA2 mutants formed filaments with cells of more homogeneous size and (iii) the tolB1 mutant did not have filaments, but rather formed swollen cells that still lacked flagella. Vesicles and large envelope excrescences still appeared at the cell surface of the pal1 mutant.

Sensitivity to antimicrobial agents

The sensitivity of the mutants towards various antimicrobial agents was analysed (Table 3). All the mutants were more sensitive to sodium cholate than the wild-type strain. In E. coli, only tolQRAB pal mutants show this phenotype (Vianney et al., 1996). The sensitivity of the ybgC1 mutant to sodium cholate can be explained by the polarity of the insertion. Consistent with this hypothesis, this phenotype could be complemented by the addition of a multicopy plasmid carrying the Er. chrysanthemi ybgCtolQRA cluster, but not when the plasmid contained only ybgC (data not shown). The ybgF1 mutant was sensitive to cholate, but resistant to the other chemical compounds tested. This phenotype could be complemented by providing the tolBpalybgF genes in trans (data not shown). The other mutants were sensitive to SDS and carbonyl cyanide m-chlorophenylhydrazone (CCCP), but resistant to vancomycin (data not shown). The tolB1 and pal1 mutants were more sensitive to chemical compounds than the tolQ2 and tolA1 mutants. However, with the exception of SDS, most differences in sensitivity were moderate.

Complementation between Er. chrysanthemi and E. coli

As shown previously, the tol-pal genes of Er. chrysanthemi were able to complement the cholate sensitivity and the motility of the E. coli tol-pal mutants. In contrast, the E. coli tol-pal genes did not complement these genes when they were introduced in the Er. chrysanthemi tol-pal mutants, even if expression of the E. coli TolA, TolB and Pal proteins could be detected by Western blotting in the Er.
chrysanthemi tolA, tolB and pal mutants. These data suggest either that the Er. chrysanthemi Tol-Pal proteins are involved in additional functions or that the E. coli Tol-Pal proteins are unable to interact appropriately with other components of the Er. chrysanthemi cell envelope.

**Virulence and survival in plant tissues of the ybgC, tolQ, tolA, tolB, pal and ybgF mutants**

The tolQ, tolA, tolB and pal mutants were strongly affected for virulence, since inoculation with these strains led to poor tissue maceration on chicory leaves (Fig. 4) or potato tubers (data not shown). Global pectate lyase activity (relative units) was strongly decreased in the macerated tissues of all the tol-pal mutants (ranging from 0.0008 to 0.008, while the activity of the wild-type strain was 0.02), decreased by two-fold in the ybgC1 mutant, but increased to 0.03 in the ybgF1 strain (data not shown). Wild-type and mutant strains were recovered from macerated tissue and observed under a phase-contrast microscope: their motility and morphology was analogous to that observed when the same strains were grown on 10% sucrose LB plates (data not shown).

**DISCUSSION**

This study was undertaken to extend information about the role of the ybgC tolQRAB pal ybgF genes in a
phytopathogenic Gram-negative bacterium. Indeed, tolQRAB pal mutations have been extensively characterized in many Gram-negative bacteria (Dennis et al., 1996; Fortney et al., 2000; Heilpern & Waldor, 2000; Llamas et al., 2000; Prouty et al., 2002). Mutants in all these genes are impaired in the so-called phenotype of outer-membrane integrity: release of periplasmic content in the extracellular medium, increased sensitivity to deleterious agents such as bile salts, motility and outer-membrane blebbing (P. putida), and entry of filamentous phage DNA (E. coli, V. cholerae).

In Er. chrysanthemi, inactivation of tolQ, tolA, tolB or pal is deleterious, since mutants in these genes grow very slowly on LB plates. They can grow well only when the medium is supplemented with 10% sugars (sucrose, glucose, maltose or lactose) or with the osmoprotectant glycine betaine, but not in the presence of glycerol or 0.1–0.3 M NaCl. Glycine betaine and sugars are non-ionic compatible solutes that can be accumulated by de novo synthesis or transport without interfering with vital cellular processes. These compounds not only confer protection against high osmolarity, but also allow protein protection (Poolman & Glaasker, 1998). The addition of sugars or betaine to the growth medium probably contributes to the osmoprotection of some envelope components. Consistent with this hypothesis, all the tol-pal mutants showed increased resistance to chemical compounds in the presence of sucrose (data not shown), and the tolQ and tolA mutants were more motile under such conditions (Table 2). The addition of sugars may also contribute to the maintenance of a turgor pressure compatible with cell viability for the Er. chrysanthemi tol-pal mutants. As in P. putida (Llamas et al., 2000), we observed that the tolB and pal mutations led to more severe phenotypes than those observed with tolQ and tolA mutants. Attempts to construct tol-pal mutants in some bacteria have been unsuccessful, and could be explained by their poor growth in classical rich media, as observed for Er. chrysanthemi tol-pal mutants (Dennis et al., 1996; Spinola et al., 1996).

Our electron microscopy observations provide a good explanation for the impairment of cell motility of the tol-pal mutants. The tolB and pal mutants lack flagella, while tolQ and tolA mutants have fewer and shorter flagella, a phenotype which suggests an alteration of flagella synthesis, polymerization and/or stability.

The Er. chrysanthemi tol-pal mutants have an altered cell morphology: some of the cells do not correctly localize the septum and the poles during division. A phenotype of filamentation has also been observed in tol-pal mutants of P. putida (Llamas et al., 2000), E. coli (Meury & Devilliers, 1999) and V. cholerae (Heilpern & Waldor, 2000), and the incorrect positioning of the septa has also been reported (Meury & Devilliers, 1999).

### Table 3. Sensitivity of Er. chrysanthemi tol-pal mutants to chemical compounds

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sodium cholate (mg ml⁻¹)</th>
<th>SDS (mg ml⁻¹)</th>
<th>CCCP (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3937</td>
<td>15</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>ybgC1</td>
<td>10</td>
<td>0.9</td>
<td>20</td>
</tr>
<tr>
<td>tolQ2</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolA1</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolA2</td>
<td>7.5</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>tolB1</td>
<td>7.5</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>pal1</td>
<td>7.5</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>ybgF1</td>
<td>10</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

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![Fig. 4. Pathogenicity of tol-pal mutant strains on chicory leaves. Bacteria (10⁷) were inoculated into scarified chicory leaves. Disease symptoms were observed after 24 h. T, scarified but not inoculated leaf.](image-url)
Er. chrysanthemi tol-pal mutants showed a reduced virulence on chicory leaves and potato tubers. Altered virulence of tolB and pal mutants has been reported in animal pathogens. In H. ducreyi, expression of Pal is required for virulence in a human model (Fortney et al., 2000). In E. coli, Pal is involved in Gram-negative sepsis (Hellman et al., 2002; Liang et al., 2005). In S. enterica, tolB mutants are attenuated in a mouse typhoid model of infection (Bowe et al., 1998). Although tolB mutants cross the gut, they are unable to cause fatal infection. This has been attributed to their inability to survive within macrophages and resist the bactericidal effects of non-immune serum. In the same screening, the authors also identified the mdoB gene as essential for fatal infection (Bowe et al., 1998). opg (or mdo) mutants have been associated with a lack of virulence in phytopathogenic bacteria such as Pseudomonas syringae and Er. chrysanthemi (Loubens et al., 1993; Mukhopadhyay et al., 1988; Page et al., 2001). Unlike the tol-pal mutants, the opgGH mutants show a complete loss of virulence, even on potato tubers, but present some phenotypes similar to those observed for the tol-pal mutants, namely reduced motility and pectate lyase production (Page et al., 2001). Another common trait between Opg and Tol-Pal is the activation of regulatory networks, such as the Rcs phosphorelay. This work further demonstrates that the Tol-Pal proteins are critical cell envelope components necessary for bacterial virulence. The low growth rate of the Er. chrysanthemi tol-pal mutants, their impaired viability, and their reduced motility and pectinase production are probably sufficient to explain the reduced virulence. It also provides further evidence that the loss of Tol-Pal functions can be deleterious for some bacterial species.

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REFERENCES


